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## **Increased HLA-DR expression and cortical demyelination in MS links with HLA-DR15**

Enz, Lukas Simon ; Zeis, Thomas ; Schmid, Daniela ; Geier, Florian ; van der Meer, Franziska ; Steiner, Guido ; Certa, Ulrich ; Binder, Thomas Martin Christian ; Stadelmann, Christine ; Martin, Roland ; Schaeren-Wiemers, Nicole

**Abstract:** **OBJECTIVE:** To investigate molecular changes in multiple sclerosis (MS) normal-appearing cortical gray matter (NAGM). **METHODS:** We performed a whole-genome gene expression microarray analysis of human brain autopsy tissues from 64 MS NAGM samples and 42 control gray matter samples. We further examined our cases by HLA genotyping and performed immunohistochemical and immunofluorescent analysis of all human brain tissues. **RESULTS:** HLA-DRB1 is the transcript with highest expression in MS NAGM with a bimodal distribution among the examined cases. Genotyping revealed that every case with the MS-associated HLA-DR15 haplotype also shows high HLA-DRB1 expression and also of the tightly linked HLA-DRB5 allele. Quantitative immunohistochemical analysis confirmed the higher expression of HLA-DRB1 in HLA-DRB1\*15:01 cases at the protein level. Analysis of gray matter lesion size revealed a significant increase of cortical lesion size in cases with high HLA-DRB1 expression. **CONCLUSIONS:** Our data indicate that increased HLA-DRB1 and -DRB5 expression in the brain of patients with MS may be an important factor in how the HLA-DR15 haplotype contributes to MS pathomechanisms in the target organ.

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# Increased HLA-DR expression and cortical demyelination in MS links with HLA-DR15

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## Abstract

### Objective

To investigate molecular changes in multiple sclerosis (MS) normal-appearing cortical gray matter (NAGM).

### Methods

We performed a whole-genome gene expression microarray analysis of human brain autopsy tissues from 64 MS NAGM samples and 42 control gray matter samples. We further examined our cases by HLA genotyping and performed immunohistochemical and immunofluorescent analysis of all human brain tissues.

### Results

HLA-DRB1 is the transcript with highest expression in MS NAGM with a bimodal distribution among the examined cases. Genotyping revealed that every case with the MS-associated *HLA-DR15* haplotype also shows high HLA-DRB1 expression and also of the tightly linked *HLA-DRB5* allele. Quantitative immunohistochemical analysis confirmed the higher expression of HLA-DRB1 in *HLA-DRB1\*15:01* cases at the protein level. Analysis of gray matter lesion size revealed a significant increase of cortical lesion size in cases with high HLA-DRB1 expression.

### Conclusions

Our data indicate that increased HLA-DRB1 and -DRB5 expression in the brain of patients with MS may be an important factor in how the *HLA-DR15* haplotype contributes to MS pathomechanisms in the target organ.

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## Glossary

**GM** = control gray matter; **HLA** = human leukocyte antigen; **MOG** = myelin oligodendrocyte glycoprotein; **MS** = multiple sclerosis; **NAGM** = normal-appearing cortical grey matter; **NeuN** = neuronal nuclei; **OLIG2** = oligodendrocyte transcription factor 2; **SNP** = single nucleotide polymorphism.

Multiple sclerosis (MS), the most common inflammatory neurologic disease affecting young adults, is a chronic autoimmune demyelinating disease of the CNS. If untreated, MS leads to disability in a substantial proportion of patients. The etiology of MS includes a complex genetic trait and several environmental risk factors, which act in concert and contribute to the main pathomechanisms including autoimmune inflammation, de- and remyelination, axonal and neuronal loss, astroglia activation, and metabolic changes.<sup>1</sup> The relative severity of these factors leads to the enormous heterogeneity of MS with respect to clinical signs, course, and response to treatment, but also pathologic composition of demyelinated lesions. The pathologic hallmark of MS is the formation of focal areas of myelin loss in the CNS. Besides the most commonly described white matter lesions, extensive gray matter lesions can be found in the MS cerebral cortex.<sup>2</sup> In addition to the well-described demyelinated gray matter lesions also diffuse gray matter abnormalities in nonlesional normally myelinated areas have been described.<sup>3–5</sup> At the molecular level, little is known about changes in normal-appearing cortical gray matter (NAGM) and gray matter lesions in MS. In the last years, several transcriptome studies of MS brain tissues have been performed, and a number of possible pathomechanisms could be identified such as mitochondrial dysfunction, metabolic changes in astrocytes, inflammation, and oxidative stress.<sup>3,6–8</sup> A limitation of all these studies is the low number of tissue samples and cases and consequently the limited statistical power. The problem is further accentuated by the heterogeneity of MS, reflected by the variable clinical course, different clinical symptoms and imaging findings, and variability in pathology. As part of our published studies,<sup>8–10</sup> we collected a large number of well-characterized human brain tissue samples from control and MS cases.

Here, we compared the expression pattern of MS NAGM with control gray matter (GM) to understand if there are alterations that may underlie or contribute to the formation of the widespread cortical lesions as an important aspect of MS pathology.

## Methods

### Tissue selection and characterization

MS and control tissue samples were provided by the UK MS Tissue Bank (UK Multicentre Research Ethics Committee, MREC/02/2/39), funded by the MS Society of Great Britain and Northern Ireland registered charity 207495, or obtained from the archives of the Institute of Neuropathology at the University Medical Centre Göttingen. Additional control

samples were provided by the Pathology Department of the University Hospital Basel. All cases were routinely screened by a neuropathologist to confirm diagnosis of MS and to exclude other confounding pathologies.<sup>11</sup> In total, 104 gray matter tissue blocks from 34 control cases and 101 NAGM tissue blocks from 51 MS cases were used for this study (table 1, further details in table e-1, [links.lww.com/NXI/A173](https://links.lww.com/NXI/A173)). Criteria of in- and exclusion are described in figure 1A. Tissues were characterized further by staining for neuronal nuclei (NeuN) (neurons), oligodendrocyte transcription factor 2 (oligodendrocytes), myelin oligodendrocyte glycoprotein (MOG) (myelin), and CD68 (microglia) (figure 1B). Cryostat sections (12  $\mu$ m) from fresh-frozen tissue blocks were stained as described before.<sup>8,10</sup> Antibodies and detailed protocols are described in table e-2, A and B ([links.lww.com/NXI/A173](https://links.lww.com/NXI/A173)).

### Ethical approvals

Ethical approvals for all human tissues used were given by the UK Multicentre Research Ethics Committee, MREC/02/2/39 for the cases from London, by the Ethics Committee of the University Hospital Basel for all cases from Basel, and by the ethical review committee of the University Medical Center Göttingen (#19/09/10) for all cases from Göttingen.

### RNA isolation and quality assessment

Total RNA from gray matter tissue was isolated using the Zymo ZR RNA Microprep Kit (Zymo Research, Irvine, CA) as described before.<sup>8</sup> Degraded (RNA integrity index < 6) and/or contaminated (260/280 nm ratio < 1.8; 230/280 nm ratio < 1.8) samples were excluded from the study.

### Microarray analysis and statistical analysis

From initially 151 NAGM and control gray matter samples, 35 were excluded due to the RNA integrity index being below the threshold of 6, 8 samples were excluded due to incongruence between the sex stated in the case reports and Y-chromosome linked gene expression, and 2 samples were excluded for being clear outliers in the principal component analysis. In total, 42 tissue samples from 14 control and 64 tissue samples from 25 MS cases were used for the gene expression analysis between NAGM and control GM (table 1 and table e-1, [links.lww.com/NXI/A173](https://links.lww.com/NXI/A173)). To minimize experimental bias, microarray experiments were performed together. All samples used for the gene expression study originated from the UK MS Tissue Bank. Gene expression profiling was performed using the Illumina complementary DNA-mediated annealing, selection, extension, and ligation assay according to the manufacturer's protocol<sup>12</sup> (Part No. 15018210, Revision history D, April 2012, Illumina, San

**Table 1** Patient data

Cases	Sex	Cause of death	p.m. time (h)	Age (a)	Disease duration (a)	MS type	HLA-DRB1* 15:01	No. of brain tissue blocks	No. of samples microarray NAGM-GM
<b>Control samples</b>									
<b>C01</b>	M	Myocardial infarction	8	70			Others	1	
<b>C02</b>	M	Cardiac failure and pneumonia	14	65			Others	5	
<b>C04</b>	F	Acute pancreatitis	20	58			15:01	6	
<b>C07</b>	M	Rectal cancer and pneumonia	9	89			Others	1	1
<b>C09</b>	F	Pneumonia	10	95			<b>15:01</b>	2	1
<b>C10</b>	F	Esophagus cancer	9	85			Others	3	
<b>C11</b>	F	Bronchopneumonia and cerebrovascular accident	9	93			Others	1	1
<b>C13</b>	M	Cardiogenic shock	21	73			Others	4	2
<b>C14</b>	M	Lung cancer, metastasized	26	77			Others	3	3
<b>C15</b>	M	Myocardial infarction	18	64			Others	4	4
<b>C17</b>	F	Congestive cardiac failure	24	84			Others	4	3
<b>C18</b>	M	Carcinoma of the tongue	22	35			<b>15:01</b>	6	4
<b>C20</b>	F	Ovarian cancer	13	60			Others	5	5
<b>C21</b>	M	Cerebrovascular accident and pneumonia	17	75			Others	4	9
<b>C22</b>	M	Prostate cancer, metastasized	22	88			Others	1	
<b>C25</b>	M	Bladder cancer and pneumonia	5	84			Others	3	4
<b>C26</b>	F	Breast cancer, metastasized	12	87			Others	1	2
<b>C27</b>	M	Renal failure and multiple myeloma	24	75			Others	1	2
<b>C28</b>	F	Cardiac failure	21	60			<b>15:01</b>	4	
<b>C29</b>	M	Pneumonia	20	60			Others	4	
<b>C30</b>	M	Pericardial tamponade	7	68			Others	4	
<b>C31</b>	M	Anaphylaxis	14	72			<b>15:01</b>	4	
<b>C32</b>	F	Pneumonia	16	71			<b>15:01</b>	4	
<b>C33</b>	F	Cardiac failure	12	83			Others	4	
<b>C37</b>	F	Pneumonia	14	72			Others	4	
<b>C38</b>	F	Pneumonia	3	88			Others	2	
<b>C39</b>	M	Acute cardiac death	10	69			<b>15:01</b>	6	
<b>C44</b>	F	Multiorgan failure	9	72			<b>15:01</b>	4	
<b>C45</b>	M	Cardiopulmonary degeneration and prostate cancer	22	77			Others	1	1
<b>C47</b>	M	Cardiac failure and acute erosive enteritis	15	53			Others	2	
<b>C48</b>	M	Asphyxia	11	61			<b>15:01</b>	2	

Continued

**Table 1** Patient data (continued)

Cases	Sex	Cause of death	p.m. time (h)	Age (a)	Disease duration (a)	MS type	HLA-DRB1* 15:01	No. of brain tissue blocks	No. of samples microarray NAGM-GM
C49	F	Cardiac failure	16	77			Others	2	
C50	M	Cardiac failure	21	75			15:01	2	
	14 F 19 M		Ø = 15.0	Ø = 73.2			10 15:01 23 others	104	42
Cases	Sex	Cause of death	p.m. time (h)	Age (a)	Disease duration (a)	MS type	HLA-DRB1* 15:01	No. of brain tissue blocks	No. of samples microarray NAGM-GM
MS samples									
M01	F	Breast cancer and pneumothorax	8	56	31	SPMS	Others	7	5
M02	F	Peritonitis	16	58	22	PPMS	Others	5	2
M03	F	NA	18	78	14	SPMS	Others	3	1
M04	F	Respiratory failure	21	42	18	SPMS	15:01	1	
M05	F	Sepsis	19	74	26	SPMS	15:01	1	
M06	F	Pneumonia	6	58	21	PPMS	15:01	6	10
M07	F	Pulmonary embolus and pneumonia	17	45	20	PPMS	Others	2	
M09	M	Aspiration pneumonia	8	75	38	SPMS	Others	1	1
M10	F	Pneumonia	8	72	41	SPMS	Others	1	2
M11	M	Pneumonia	26	66	31	SPMS	Others	1	
M12	F	NA	11	69	31	NA	15:01	3	
M13	M	Pneumonia	11	63	39	SPMS	Others	2	
M14	F	Pneumonia	9	77	31	PPMS	Others	4	3
M15	F	MS	15	51	21	SPMS	Others	2	2
M16	F	Adenocarcinoma of unknown primary	6	56	17	PRMS	15:01	1	1
M17	F	Respiratory infection	10	49	19	SPMS	Others	1	
M18	F	Pneumonia	13	66	30+	NA	15:01	3	
M20	F	Pneumonia	21	86	56	NA	Others	2	
M21	F	Pneumonia	11	86	36	SPMS	Others	1	
M22	F	MS	21	77	22	PPMS	15:01	1	1
M23	F	Lung cancer, metastasized	5	78	42	SPMS	15:01	4	5
M24	F	Renal failure	31	49	18	SPMS	Others	2	1
M26	F	Bowel blockage and heart failure	24	71	35	SPMS	15:01	1	
M27	F	Pneumonia	9	49	25	SPMS	Others	1	
M28	F	Pneumonia	22	54	20	SPMS	15:01	3	
M30	F	Pneumonia	7	77	21	SPMS	Others	2	1
M31	M	Urinary tract infection and MS	12	53	11	SPMS	Others	1	

Continued

**Table 1** Patient data (*continued*)

Cases	Sex	Cause of death	p.m. time (h)	Age (a)	Disease duration (a)	MS type	HLA-DRB1*15:01	No. of brain tissue blocks	No. of samples microarray NAGM-GM
M32	F	Pneumonia	18	39	21	PRMS	<b>15:01</b>	1	4
M33	M	Pneumonia	19	38	17	PRMS	Others	2	1
M34	M	NA	9	92	54	PPMS	<b>15:01</b>	1	2
M36	M	Pneumonia	16	44	16	SPMS	<b>15:01</b>	2	
M40	M	Respiratory failure	10	40	9	SPMS	<b>15:01</b>	3	
M42	F	MS	12	50	31	SPMS	Others	2	
M43	F	Pneumonia	12	34	NA	SPMS	<b>15:01</b>	1	
M44	F	Small bowel obstruction	13	80	36	SPMS	Others	2	1
M46	F	Multiorgan failure and sepsis	28	45	6	SPMS	<b>15:01</b>	2	3
M47	M	Intestinal obstruction	12	37	27	PPMS	Others	2	
M48	F	Pneumonia	24	78	47	SPMS	Others	1	2
M51	F	Pneumonia	12	59	27	PPMS	Others	3	2
M52	M	Pneumonia	24	45	25	SPMS	<b>15:01</b>	1	
M53	F	Sepsis and pneumonia	16	47	17	SPMS	<b>15:01</b>	4	4
M54	M	MS	9	45	18	SPMS	<b>15:01</b>	1	
M55	F	MS	26	37	17	SPMS	Others	2	
M56	F	Pneumonia	22	88	32	PPMS	<b>15:01</b>	1	2
M57	F	COPD	17	58	16	PPMS	Others	2	3
M58	F	MS	7	80	37	SPMS	<b>15:01</b>	1	
M59	F	MS	13	42	11	SPMS	<b>15:01</b>	2	
M60	F	Respiratory failure	9	59	4	PPMS	<b>15:01</b>	2	3
M61	M	Pancreatic cancer	10	61	26	SPMS	<b>15:01</b>	1	2
<b>37 F 12 M</b>			<b>Ø = 14.8</b>	<b>Ø = 59.9</b>	<b>Ø = 25.5</b>	<b>32 SPMS 11 PPMS 3 PRMS 3 NA</b>	<b>24 15:01 25 others</b>	<b>101</b>	<b>64</b>

Abbreviations: COPD = chronic obstructive pulmonary disease; GM = control gray matter; NA = not available; NAGM = normal-appearing cortical gray matter; p.m. time = postmortem time (hours); PPMS = primary progressive multiple sclerosis; PRMS = progressive-relapsing multiple sclerosis; SPMS = secondary progressive multiple sclerosis.

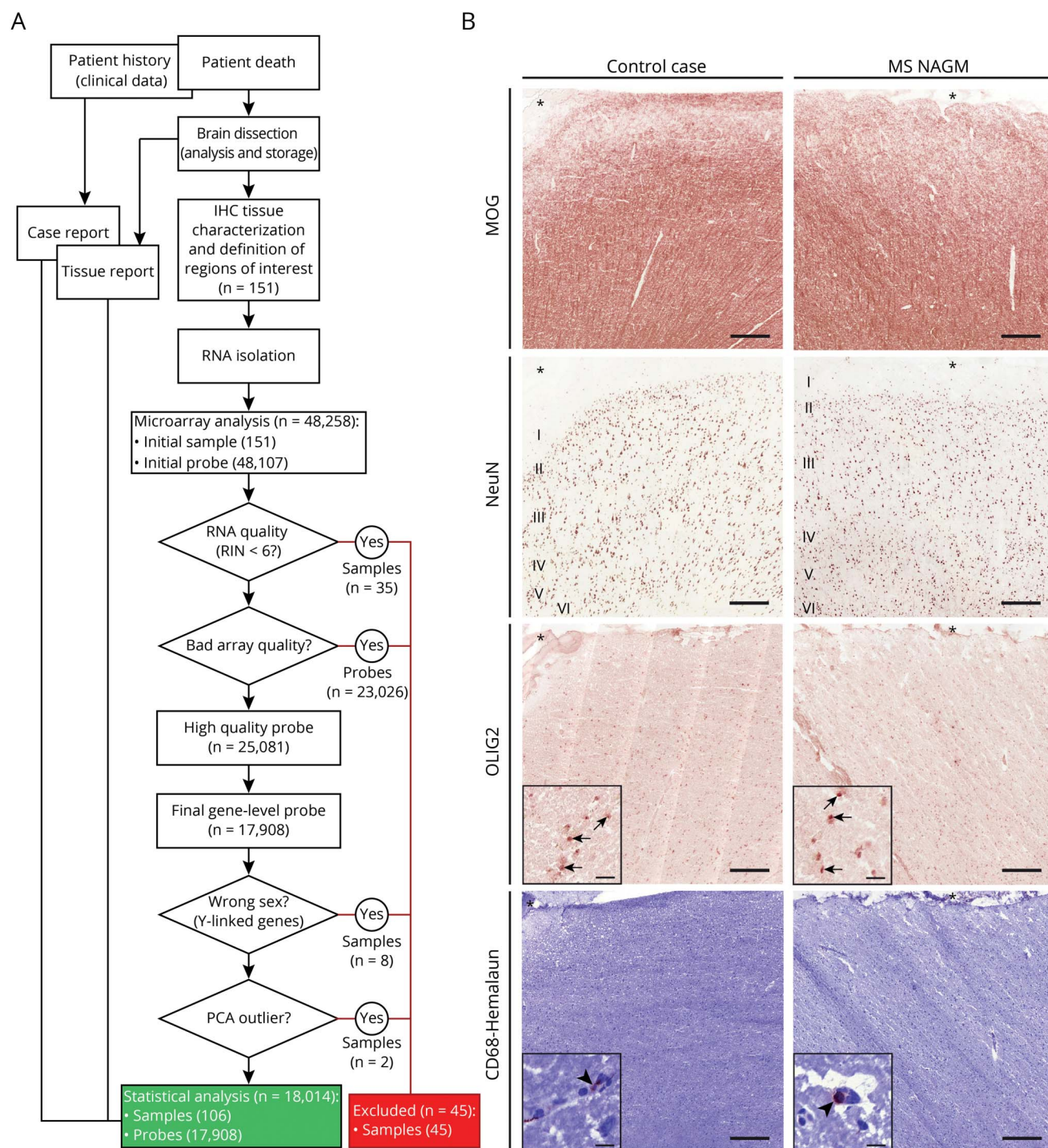
The table shows the patient characterization and the use of the patient samples in the different experiments. A more detailed table is given in the supplements (table e-1, [links.ww.com/NXI/A173](https://www.ww.com/NXI/A173)). HLA-DRB1\*15:01 is highlighted in bold.

Diego, CA). BeadChips were scanned by the iScan Array scanner (Illumina). All subsequent data analyses were performed using the statistical software R (R core development Team 2008; R version 3.5.0). Specifically, the Bioconductor packages beadarray (version 2.30.0) and illuminaHumanWGDASLv4.db (version 1.26.0) were used for reading-in data files and for probe annotation (probes  $n = 48,107$ ). Between-array normalization was performed by variance stabilizing transformation followed by a quantile normalization using functions from the Bioconductor

package lumi (version 2.32.0). Only probes mapping to an ENTREZ gene ID were retained. Probes with quality status “bad” were removed. “Bad quality probes” are probe matches repeat sequences, intergenic or intronic regions, or is unlikely to provide specific signal for any transcript (according to illuminaHumanWGDASLv4 annotation). Because the resulting probes ( $n = 25,081$ ) were still not unique, we selected the probe with the highest variance across all samples, neglecting sample values, which fall into the expression range of negative control probes. This way, each gene is



**Figure 1** Tissue processing for microarray and tissue characterization



(A) Flowchart to illustrate the process from the patient's death to statistical analysis of the gene expression microarray. After dissection of the brain and exclusion of confounding pathologies, the tissue blocks were sent to Basel, Switzerland. There, an immunohistochemical characterization was performed, any tissue with bad preservation was excluded, and regions of interest were selected. After RNA isolation, the RIN was measured, and samples with RIN smaller than 6 were excluded. Sample mix up was checked by wrong sex and by principal component analysis. (B) Representative images of control cortical gray matter (case C30) and MS NAGM (case MS08, asterisk delineates the meninges, I–VI indicate the 6 neuronal layers). NAGM was defined as no loss of MOG, NeuN, or OLIG2 (inset, arrows) staining compared with control cases and no increase in CD68 compared with controls; i.e., occasional CD68<sup>+</sup> staining intra- or perivascular (inset, arrowheads) and nearly no CD68<sup>+</sup> staining in the tissue. Scale bars: 250  $\mu$ m; inset Olig2: 20  $\mu$ m; inset CD68: 10  $\mu$ m. IHC = immunohistochemistry; MOG = myelin oligodendrocyte glycoprotein; NAGM = normal-appearing cortical gray matter; NeuN = neuronal nuclei; OLIG2 = oligodendrocyte transcription factor 2; PCA = principal component analysis; RIN = RNA integrity index.

represented by the probe, which contains most information on potential expression differences, but ignores probes, which appear artificially regulated due to false-negative

regulation introduced by single nucleotide polymorphisms (SNPs). This strategy gave rise to 17,908 unique gene-level probes.

## HLA genotyping

Human leukocyte antigen (HLA) genotyping was performed by Histogenetics (NY). Allelic variants were typed by sequencing at high resolution (3-field). Alleles bearing suffix “G” in the A locus have identical sequences in exon 2 and exon 3 antigen recognition sites. Alleles bearing suffix “G” in the DRB locus have identical sequences in exon 2 antigen recognition sites. Genotypes are shown in table e-3 ([links.lww.com/NXI/A173](https://links.lww.com/NXI/A173)).

## Immunofluorescence colocalization

Immunofluorescent colocalization was performed as described before (table e-2A, [links.lww.com/NXI/A173](https://links.lww.com/NXI/A173)).<sup>8,10</sup> As tissue preservation is not optimal in fresh-frozen tissues, further paraffin-embedded tissue blocks were stained for colocalization. Paraffin-embedded tissue sections (2–3  $\mu\text{m}$ ) were deparaffinized in xylene, rehydrated, and transferred to 3%  $\text{H}_2\text{O}_2$  in phosphate buffered saline (PBS) for 20 minutes at 4°C to block the endogenous peroxidase. After 3 washing steps with PBS, the sections were incubated with blocking buffer (10% fetal calf serum in PBS) for at least 20 minutes to reduce unspecific antibody binding. Primary antibodies (table e-2A) were diluted in blocking buffer and incubated overnight at 4°C and then washed 3 times with PBS. Secondary antibodies were incubated for 1–2 hours (table e-2A).

## Histologic quantification

HLA-DRB1 protein expression was measured with Fiji (image processing package including ImageJ)<sup>13</sup> using the count objects algorithm with the following parameters: lower threshold 0, upper threshold 120 in green channel of red-green-blue color space, size of objects  $10\ \mu\text{m}^2$ – $100\ \mu\text{m}^2$ , and circularity 0.25–1.00. Cortical lesions were defined as areas with complete loss of anti-MOG staining or areas with reduced myelin density clearly demarcated from surrounding normal-appearing tissue. Only cortical gray matter ranging from the white matter to the meninges and with all 6 neuronal layers visible in the adjacent NeuN staining was used. NAGM and gray matter lesion areas were outlined in Adobe Photoshop CS6 (version 13.0 x64; Adobe Systems, CA), exported, and evaluated for area (in squared millimeters) in ImageJ software (references 13 and 14, version 1.51s, Fiji distribution, NIH, Maryland). A schematic drawing is shown in figure 4C.

## Statistical analysis

All statistical analyses were performed using R.<sup>15</sup> A *p* value or false detection rate-adjusted *p* value smaller than 0.05 was considered statistically significant. Expression data were analyzed using R and the Bioconductor package limma (version 3.36.5). Statistical analysis was performed using a linear model with disease group and sex as factors. Because some patients contributed multiple tissue samples (tissue blocks), we additionally distinguished these “technical” replicates from true biological replicates (patients) in the model to avoid a potential inflation of significance by pseudoreplication. Specifically, the duplicateCorrelation function of the limma package was used to estimate a consensus correlation between technical replicates

and this value together with patient ID as a block factor entered into the model fit function. To test the correlation between HLA-DRB1 and HLA-DRA gene expression, a linear model was used (figure 2J). To test the influence of the *HLA-DRB1\*15:01* genotype and the HLA-DRB1 gene expression on the demyelinated gray matter lesion area per total gray matter area, *p* values were derived from a linear model weighted by number of tissue blocks per patient (figure 4D). For all other statistical tests, a 2-sided Welch *t* test was used.

## Data availability

The gene expression data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus,<sup>16</sup> accession number GSE131282, [ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE131282](https://ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE131282).

## Results

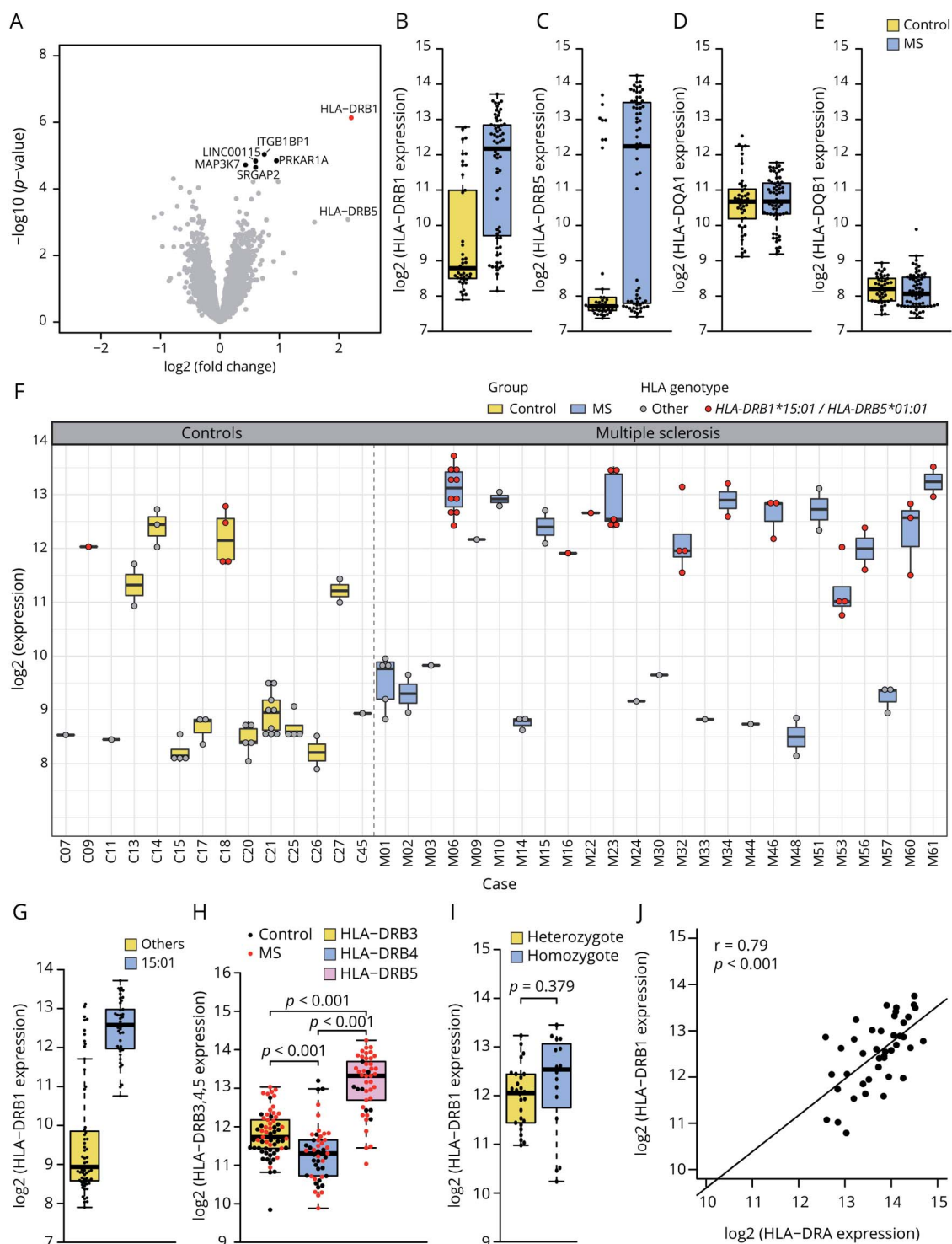
### HLA-DRB1 is significantly upregulated in MS NAGM compared with control GM

We investigated the gene expression in MS NAGM and control cases. All tissues were characterized histopathologically, and tissues with signs of possible confounding pathologies were excluded (figure 1A). As a result, only tissues without signs of demyelination, neuronal degeneration, oligodendrocyte loss, and without signs of inflammation such as microglia activation and macrophage infiltration were included in the microarray study (figure 1B). Differential gene expression analysis between MS NAGM and control GM revealed HLA-DRB1 as the most significant differentially regulated gene (figure 2A) (fold-change [FC] = 4.62, adj. *p* value = 0.013). Besides HLA-DRB1, we detected a trend toward upregulation for the integrin subunit beta-1-binding protein 1 (ITGB1BP1), protein kinase cAMP-dependent type 1 regulatory subunit alpha (PRKAR1A), long intergenic nonprotein coding RNA 115 (LINC00115), mitogen-activated protein kinase kinase kinase 7, also known as TGF1 (MAP3K7), and SLIT-ROBO Rho GTPase activating protein 2 (SRGAP2) (figure 2A).

Further analysis of HLA-DRB1 expression showed that the distribution of HLA-DRB1 expression was bimodal within both the MS and the control group (figure 2B). Of special interest is that the majority of individuals with high HLA-DRB1 expression were in the MS group. As HLA genes often show tight linkage disequilibrium patterns,<sup>17</sup> we further investigated the expression of DRB5, DQA1, and DQB1, as certain alleles of these genes were reported to form a tight linkage group within the *DR15* haplotype.<sup>18</sup> The MS-associated *DR15* haplotype consists of 5 alleles, namely *DRA\*01*, *DRB1\*15:01*, *DRB5\*01:01*, *DQA1\*01:02*, and *DQB1\*06:02*. Although not significantly differentially expressed between MS and control cases (FC = 4.45, adj. *p* value = 0.529), we also identified a bimodal distribution for HLA-DRB5 (figure 2C). HLA-DQA1 and HLA-DQB1 expression levels were normally distributed (figure 2, D and E), with HLA-DQB1 expression at the lower detection limit.



**Figure 2** Differential gene expression in MS NAGM vs control case cortical gray matter



(A) Volcano plot of the differential gene expression analysis between MS NAGM and control gray matter (GM) revealed HLA-DRB1 as the most significant differentially regulated gene (FC = 4.62, adj.  $p$  value = 0.013, marked in red). Differentially expressed genes with an adjusted  $p$  value between 0.05 and 0.1 are marked in black. This was the case for ITGB1BP1 (FC = 1.67, adj.  $p$  value = 0.065), PRKAR1A (FC = 1.93, adj.  $p$  value = 0.065), LINC00115 (FC = 1.5, adj.  $p$  value = 0.065), MAP3K7 (FC = 1.34, adj.  $p$  value = 0.067), and SRGAP2 (FC = 1.5, adj.  $p$  value = 0.067). Boxplots show the  $\log_2$  gene expression of HLA-DRB1 (B), HLA-DRB5 (C), and HLA-DQA1 (D) and HLA-DQB1 (E) between MS NAGM and GM. HLA-DRB1 is significantly differentially expressed between MS NAGM and GM (B). Both, HLA-DRB1 (B) and HLA-DRB5 (C) show a bimodal expression pattern in MS and control tissue, whereas the expression of HLA-DQA1 (D) was normally distributed within the sample groups, and HLA-DQB1 (E) was at the lower detection limit. (F) Boxplots show  $\log_2$  of the HLA-DRB1 gene expression in all tissue samples and in all cases used for the microarray analysis. All cases with the *HLA-DRB1\*15:01* genotype show a high HLA-DRB1 expression (red dots). (G) HLA-DRB1 expression between samples from cases carrying the *HLA-DRB1\*15:01* or other *HLA-DRB1* alleles. Boxplot shows that all *HLA-DRB1\*15:01* tissue samples belong to the HLA-DRB1 high expresser group. (H) HLA-DRB3, 4, and 5 expression of all samples. (I) Comparison of hetero- and homozygote carriers of the *HLA-DRB1\*15:01* allele. (J) Correlation of HLA-DRB1 with HLA-DRA within the *HLA-DRB1\*15:01* positive samples. (H and I)  $p$  Values are derived from a 2-sided Welch  $t$  test. (J)  $p$  Value is derived from a linear model.

## High cortical HLA-DRB1 expression is associated with the *HLA-DRB1\*15:01* haplotype

The bimodal HLA-DRB1 expression pattern prompted us to investigate whether HLA-DRB1 expression in all tissue samples from 1 individual shows this mode of expression. This analysis revealed that single cases either expressed HLA-DRB1 at high or low levels in both MS and control cases (figure 2F). As *HLA-DRB1\*15:01* is strongest associated with MS risk<sup>19,20</sup> and HLA-DRB5 showed a similar expression distribution, we genotyped all cases for *HLA-DRB1*, 3, 4, and 5 at a 3-field resolution (table e-3, [links.lww.com/NXI/A173](https://links.lww.com/NXI/A173)). As expected, we detected a trend toward higher frequency of the *HLA-DRB1\*15:01* allele among the MS cases compared with control cases (Fisher exact test,  $p = 0.083$ , OR = 4.5, 95% CI = [0.8–50.2]).<sup>21</sup> Gene expression analysis based on the *HLA-DRB1* genotype revealed that the bimodal distribution was linked to the *HLA-DRB1* genotype with individuals with the *HLA-DRB1\*15:01* allele always showing high transcriptional expression of HLA-DRB1 ( $n = 106$  samples, figure 2, F and G). In contrast to HLA-DRB1, which is expressed in every case, only 11 MS and 1 control case turned out to carry the *HLA-DRB5* gene (table e-3). As expected, all individuals genotyped positively for *HLA-DRB5\*01:01* allele were also positive for *HLA-DRB1\*15:01* (table e-3). Compared with HLA-DRB3 and -DRB4 alleles in other DR haplotypes, *HLA-DRB5\*01:01* always showed a higher expression ( $p < 0.001$ ,  $df = 79.33$ , for DRB3, figure 2H;  $p < 0.001$ ,  $df = 89.58$  for DRB4, figure 2H). Notably, there were no significant differences in HLA-DRB1 gene expression levels between hetero- and homozygotic carriers of the *HLA-DRB1\*15:01* allele ( $p = 0.379$ ,  $df = 22.01$ , figure 2I). Beside the cases carrying the *HLA-DRB1\*15:01* allele, 5 MS and 3 control cases also showed high HLA-DRB1 expression (figure 2F). Of these cases, one MS case was heterozygote for the *HLA-DRB1\*04:01* allele, and another case was heterozygote for the *HLA-DRB1\*08:01:01G* allele. Of interest, both alleles have been associated with risk of MS.<sup>19,20</sup> All 3 control cases were heterozygote for the *HLA-DRB1\*03:01* allele, also previously shown to be associated with MS<sup>19</sup> (table e-3, risk genes marked in bold). Of the other 3 MS cases with high HLA-DRB1 expression, 2 did not carry a MS-associated allele (*HLA-DRB1\*01:01:01*, *\*01:01:01G*, *\*01:03*, and *\*07:01:01G*), and in 1 case, genotyping failed.

We did not detect any systematic differences between the *HLA-DRB1\*15:01* cases compared with the other cases concerning age at death, age at disease onset, and disease duration (figure e-1, A–C, [links.lww.com/NXI/A173](https://links.lww.com/NXI/A173)). Also, we did not detect any differences between the HLA-DRB1 high expressing cases compared with the low expressing cases concerning age at death, age at disease onset, and disease duration (figure e-1, D–F).

## High HLA-DRB1 expression correlates with high expression of HLA-DRA

Functional HLA-DR molecules are heterodimers of a DRA-encoded alpha chain and a beta chain encoded by *DRB1* or *DRB3,4,5*, respectively. Therefore, we investigated whether

high HLA-DRB1 expression correlates with high HLA-DRA gene expression in *HLA-DRB1\*15:01* carriers. Indeed, high HLA-DRB1 gene expression correlated with high HLA-DRA ( $r = 0.79$ ,  $p < 0.001$ ) gene expression in *HLA-DRB1\*15:01* cases, supporting the idea of a biologically functional upregulation of MHCII in MS NAGM of *HLA-DRB1\*15:01* cases (figure 2J).

## HLA-DRB1 is expressed by microglia in human cortical gray matter

To determine which cell types are expressing HLA-DRB1 in NAGM, a confocal immunofluorescence colocalization analysis of fresh-frozen and paraffin-embedded human brain tissues was performed (figure 3). We detected that HLA-DRB1 colocalized with microglia in MS NAGM and control GM (figure 3A). No colocalization could be detected in astrocytes (figure 3B), neurons (figure 3C), oligodendrocytes (figure 3D), or blood vessels (figure 3E).

## HLA-DRB1 protein expression is elevated in *HLA-DRB1\*15:01*-positive cases

Quantitative immunohistochemical analysis was performed to determine whether HLA-DRB1 gene and protein expression are associated with each other (figure 4A). We detected a higher protein expression in high vs low HLA-DRB1 gene expressers ( $p = 0.052$ ,  $df = 46.9$ ,  $n = 49$ ) (figure 4B) and a trend toward higher expression in MS and control cases carrying the *HLA-DRB1\*15:01* allele compared with non-carriers ( $p = 0.097$ ,  $df = 60.5$ ,  $n = 74$ ). HLA-DRB1 protein expression varied considerably from case to case (figure 4B).

## *HLA-DRB1\*15:01* genotype and increased expression of HLA-DRB1 is associated with increased cortical demyelination

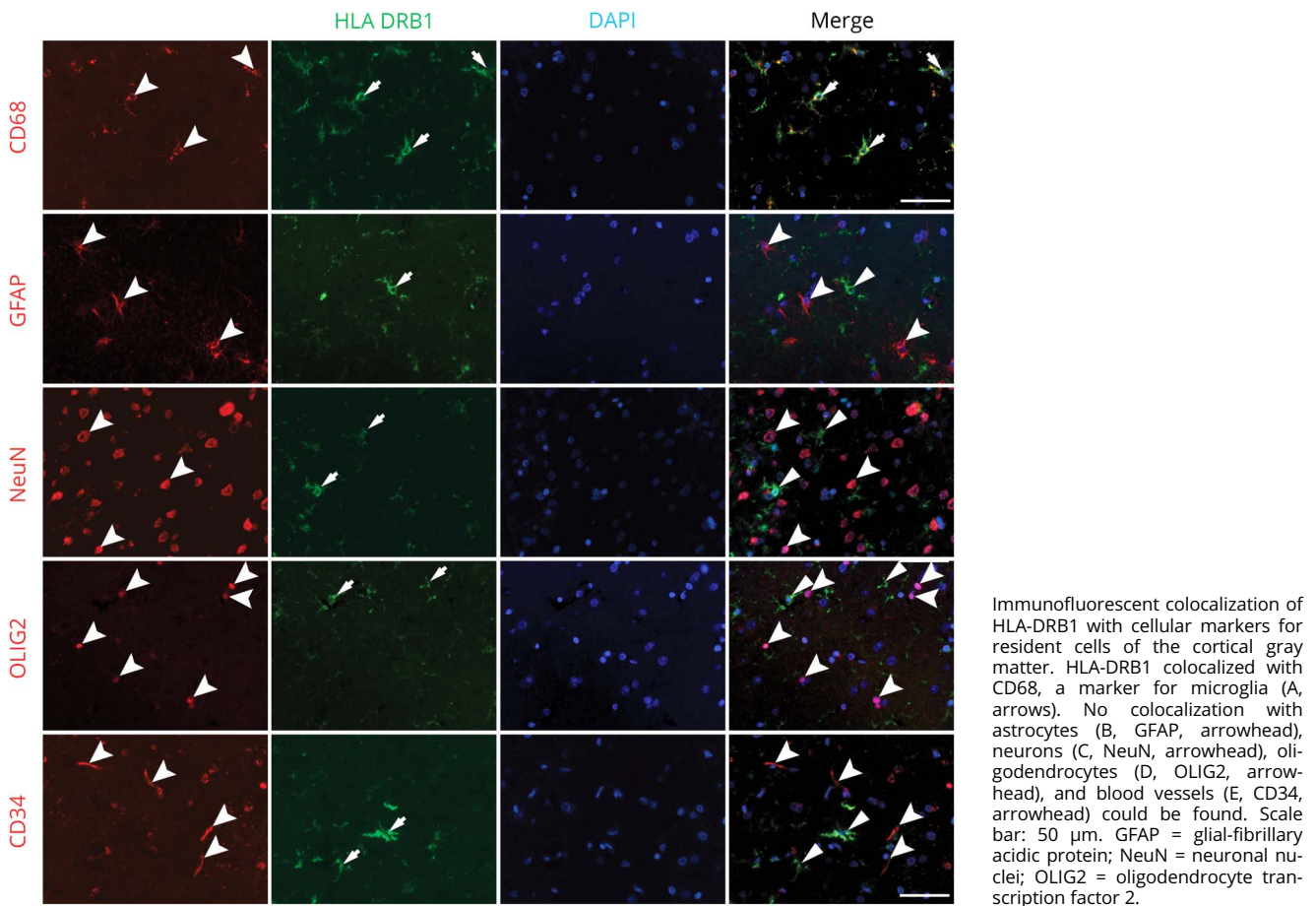
We hypothesized that the *HLA-DRB1\*15:01* genotype and the increased HLA-DRB1 gene- and protein expression might be related to meningeal inflammation and cortical microglia activation, previously described in MS,<sup>22</sup> and hence might also correlate with increased levels of cortical demyelination.

We thus investigated all available tissue blocks from MS cases with cortical lesions (table e-1, [links.lww.com/NXI/A173](https://links.lww.com/NXI/A173)) and stained for MOG, delineated cerebral cortical area containing all 6 layers (identified by NeuN) (figure 4C), and quantified the fraction of demyelinated vs whole cortical area (figure 4D). Areas of demyelination in MS cases carrying the *HLA-DRB1\*15:01* allele, and respectively cases with a high HLA-DRB1 gene expression, were significantly larger than in *HLA-DRB1\*15:01*-negative cases ( $p = 0.052$ ,  $df = 23$ ,  $n = 25$ ) and, respectively, cases with low HLA-DRB1 gene expression ( $p = 0.014$ ,  $df = 21$ ,  $n = 23$ ) (figure 4D).

## *HLA-DRB1\*15:01* carrier status associates with higher expression of 9 genes in cortical NAGM in both MS and controls

We investigated the effect of the *HLA-DRB1\*15:01* allele by analyzing the differential gene expression data after grouping

**Figure 3** HLA-DRB1 colocalization in MS cortical gray matter



the cases into *DRB1\*15:01* positive or negative. We detected 9 genes to be differentially regulated. Most interestingly, our data show an upregulation of interleukin 18 receptor 1 (*IL18R1*; FC = 1.73, adj. *p* value = 0.004) and leukocyte immunoglobulin like receptor B1 (*LILRB1*; FC = 1.54, adj. *p* value = 0.032). The highest fold change was detected for long intergenic nonprotein coding RNA 01119 (*LINC01119*, FC = 4.35, adj. *p* value < 0.0001). We further detected differentially expressed transcripts of protein O-fucosyltransferase 2 (*POFUT2*; FC = 1.86, adj. *p* value < 0.001), G protein subunit beta 5 (*GNBS*; FC = -2.20, adj. *p* value = 0.003), epithelial stromal interaction 1 (*EPSTI1*; FC = 1.50, adj. *p* value = 0.005), DExD/H-box helicase 60 (*DDX60*; FC = 1.25, adj. *p* value = 0.010), N-acetylneuraminic acid phosphatase (*NANP*; FC = 1.47, adj. *p* value = 0.012), and kinesin family member 25 (*KIF25*; FC = -1.37, adj. *p* value = 0.049).

## Discussion

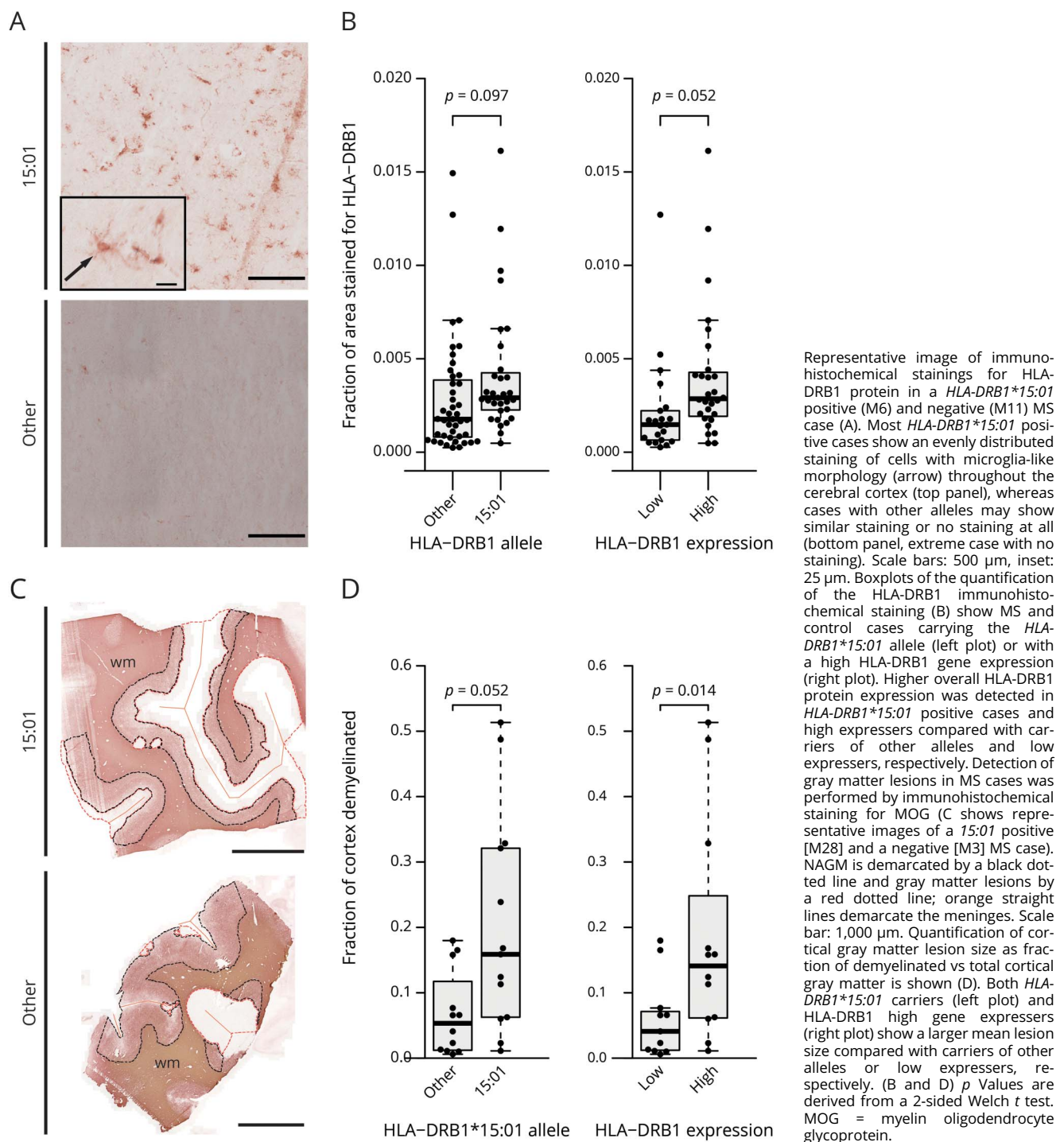
Our results demonstrate that HLA-DRB1 is significantly higher expressed in MS NAGM and shows a bimodal distribution with more MS cases showing a high expression

compared with control cases. Genotyping of the HLA locus revealed an almost exclusive high expression of all *HLA-DRB1\*15:01* allele carriers and of a few other MS-associated risk alleles. Consistent with the gene expression analysis, HLA-DRB1 protein expression is increased in *HLA-DRB1\*15:01*-positive cases in gray matter on microglia based on immunofluorescence colocalization. The *HLA-DRB1\*15:01* genotype and high HLA-DRB1 gene expression are associated with larger gray matter lesions in MS. Furthermore, it is important to note that the second DR allele, *DRB5\*01:01*, which is tightly linked with *DRB1\*15:01* in the MS-associated DR haplotype is also expressed at higher levels.

These findings hint at a link between the strongest genetic risk factor for MS and an important pathologic hallmark, namely demyelinated lesions in the cerebral cortex. We hypothesize that the *HLA-DRB1\*15:01* genotype and the increased HLA-DRB1 and-DRB5 gene expression together with meningeal and parenchymal inflammation may lead to larger demyelinated lesions. The correlation between inflammation, cortical demyelination, and the *HLA-DRB1\*15* allele has already been described in autopsy tissue



**Figure 4** HLA-DRB1 protein expression in MS and control cases and cortical gray matter lesion size in MS cases associate with *HLA-DRB1\*15:01* allele



by Yates et al.,<sup>23</sup> who have detected more parenchymal, perivascular, and meningeal T-cell inflammation and larger motor cortical lesions in *HLA-DRB1\*15* carriers compared with carriers of other alleles. Although MRI detection of cortical gray matter lesion is improving, widespread subpial demyelination is still difficult to detect.<sup>24</sup> A recent MRI study of 85 patients with MS did not reveal a statistically significant

difference between *HLA-DRB1\*15:01* carriers and non-carriers; however, the authors point out the limited power due to the small number of cases.<sup>25</sup> Furthermore, meningeal inflammation and follicle-like structures in the meninges have been linked to microglia activity<sup>22</sup> and larger subpial cortical gray matter lesions.<sup>26</sup> Regarding expression of the 2 *DR15* alleles, higher messenger RNA expression of

*DRB5\*01:01* compared with *DRB1\*15:01* in MS lesions and normal-appearing white matter have already been observed previously supporting our findings; however, much fewer cases and neither gray matter nor the extent of demyelination had been studied.<sup>27</sup>

HLA class II molecules present processed peptides to CD4<sup>+</sup> T lymphocytes. In MS, the strongest genetic association maps to the *HLA-DRB1* gene, whereas the association with *DRB5\*01:01* has so far largely been ignored because the SNPs, which have been used for determining DR types from SNP typing, are not sufficiently tightly spaced in the *HLA* region on chromosome 6 to allow assignment of the specific *HLA-DR3\**, *-4\**, and *-5\** alleles.<sup>20</sup> The *HLA-DRB1\*15:01* allele was reported to increase the risk for developing MS about threefold.<sup>28</sup> The higher *HLA-DRB1* expression in the cortical gray matter in *HLA-DRB1\*15:01* cases may therefore be involved in local activation of autoreactive CD4<sup>+</sup> T cells. For CD4<sup>+</sup> T-cell activation to occur, an interaction between the T-cell receptor and major histocompatibility complex (MHC) class II/peptide (pMHC) complexes is required.<sup>29</sup> The amount of antigen loaded on MHC class II molecules of antigen-presenting cells and the level of MHC class II expression determine the activation of CD4<sup>+</sup> T-cell activation, and higher pMHC ligand densities enhance this process.<sup>30</sup> Similar to the ectopic expression of HLA class II in autoimmune thyroid disease,<sup>31</sup> higher *HLA-DRB1* expression by microglia in the brain may therefore affect the pMHC concentration and consequently lead to an increased probability of T-cell activation and brain inflammation. This hypothesis is further supported by the finding that the level of *HLA-DR* expression in transgenic mice is an important prerequisite for developing spontaneous experimental autoimmune encephalomyelitis.<sup>32</sup>

Besides their expression levels, the nature of the peptide repertoire that is presented by the 2 *HLA-DR* alleles in the MS-associated *DR15* haplotype, i.e., *DRB1\*15:01* and *DRB5\*01:01*,<sup>27</sup> probably also plays an important role for the activation of autoreactive T cells.<sup>33,34</sup> Finally, autoreactive- and virus-specific, brain-infiltrating CD4<sup>+</sup> T cells may recognize peptides in the context of both *DR* alleles of the *DR15* haplotype, indicating that the expression of the 2 *DR* molecules may increase the likelihood of T-cell activation further.<sup>35,36</sup>

A bimodal expression pattern of *HLA-DRB1* and *-DRB5* has been described in lymphoblastoid cell lines.<sup>37</sup> The authors analyzed expression quantitative trait loci in the tag of the *HLA-DRB1\*15:01* allele associating with high *HLA-DRB1*, *DRB5*, and *DQB1* gene expression. They concluded that a higher gene expression alone does not sufficiently explain the MS-associated risk of the *HLA-DRB1\*15:01* allele. At present, it is not clear which molecular pathomechanisms are responsible for the high vs lower expression in some individuals, but differences in gene regulation, for instance by different activation of the class II transactivator, is one possibility.<sup>38</sup>

Regarding the potential functional involvement of the *DRB1\*15:01* haplotype, the higher *DRB1*- and *DRB5* expression in MS brains raises questions beyond peptide presentation to autoreactive T cells. The brain is considered an immune privileged site, and besides shielding of the CNS from the peripheral immune system via specialized barriers, low MHC expression is considered an important aspect of this CNS immune privilege.<sup>39</sup> Aberrant and increased expression of *HLA-DRB1* and *-DRB5* may contribute to breaking immune tolerance in this tissue that is exquisitely vulnerable to damage and endowed with cells that are terminally differentiated and not replaceable. The interpretation of recent genome-wide association studies in MS, which found almost exclusively immune system-related genes, has been that MS develops from outside, i.e., the peripheral immune system, in as opposed to starting by damage within the CNS and then involving peripheral immune cells, i.e., inside-out hypothesis.<sup>40</sup> Our data, although preliminary and not addressing functional aspects in human brain tissue, might indicate that increased *HLA-DR* expression in the brain, if it preceded peripheral immune T-cell activation, could play a role both within and later also outside the brain.

Regarding gene expression in cortical gray matter, several studies have been performed with a relative small set of NAGM tissue samples<sup>3,6,7</sup> including ours.<sup>8</sup> The present study with a much larger number of cases and tissues did not show the same gene expression alterations as the previous studies. Possible reasons are the different gene expression platforms, tissue preparation (fresh frozen vs paraffin embedded), the statistical methods, and, most relevant, differences in the patient samples, which is a critical aspect in a heterogeneous disease like MS.

The gene expression analysis further revealed 9 genes to be differentially expressed in *HLA-DRB1\*15:01*-positive vs -negative samples. Among the detected genes, *IL18R1* gene and protein expression have previously been shown to be elevated in MS in CSF and peripheral blood mononuclear cells compared with controls.<sup>41</sup> The highest fold change was detected for *LINC01119*, a long intergenic non-protein-coding RNA. Research on noncoding RNAs is rapidly evolving, and some members have already been shown to play a role in immune system regulation.<sup>42</sup> We further detected *LILRB1*, a receptor for class I MHC antigens expressed by different leukocyte lineages that may downregulate monocyte activation signals. *EPSTI1*<sup>43</sup> and *DDX60*<sup>44</sup> have previously been shown to be differentially regulated on interferon signaling in *HLA-B27*-transgenic rats, an animal model developing spontaneous autoimmune-mediated multisystem inflammatory disease.<sup>45</sup> The other genes we detected were *POFUT2*,<sup>46</sup> *GNBS*,<sup>47</sup> *NANP*,<sup>48</sup> and *KIF25*.<sup>49</sup> To the best of our knowledge, these genes have not been described to date in the context of MS or *HLA-DRB* as possibly linked to the *HLA-DRB1\*15:01* allele. The power of this particular analysis is however limited by the sample heterogeneity and needs



further experimental investigation to evaluate the possible impact of the detected differential expression.

In conclusion, our results demonstrate elevated DR expression in the cortical gray matter of a subset of patients with MS positive for the *HLA-DR15* haplotype and rarely also other DR types. HLA-DRB1 expression by microglia in the brain might play a role as vulnerability factor to develop or sustain MS. Further studies on DR expression in the brain, its causes, and consequences would therefore be of great interest for a better understanding of MS pathogenesis.

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<b>Daniela Schmid, PhD</b>	University and University Hospital Basel, Switzerland	Author	Sample selection and characterization
<b>Florian Geier, PhD</b>	University and University Hospital Basel, Switzerland	Author	Data analysis and interpretation and statistics
<b>Franziska van der Meer, PhD</b>	University Medical Center Göttingen, Germany	Author	Immunofluorescence colocalization
<b>Guido Steiner, PhD</b>	Roche Innovation Center Basel, Switzerland	Author	Microarray experiment design
<b>Ulrich Certa, PhD</b>	Roche Innovation Center Basel, Switzerland	Author	Study design
<b>Thomas Martin Christian Binder, MD</b>	Institute of Clinical Transfusion Medicine, Hospital Braunschweig, Germany	Author	Manuscript preparation
<b>Christine Stadelmann, MD</b>	University Medical Center Göttingen, Germany	Author	Immunofluorescence colocalization
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<b>Nicole Schaeren-Wiemers, PhD</b>	University and University Hospital Basel, Switzerland	Author	Study design, data analysis and interpretation, and manuscript preparation

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